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Shalaby, Shalaby W., Ph.D.

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Anderson, South Carolina 29625

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13. ABSTRACT *(Maximum 200 words)*

The three segments of the planned studies, adhesive skin wound augmentation (ASWA), burn wound healing (BWH), and hemostatic sealants (HS) were successfully completed. Results on ASWA led to the identification of an absorbable extrudable gel-former which can be used in conjunction with skin stapling to reduce the number of staples needed to approximate wound edges by about half, while increasing significantly the percent regain in wound strength and controlling scar formation. Relative to BWH, a representative gel-former was found to assist in accelerating the healing process while reducing scar formation as indicated by the extent of epithelization and width of dermal fibroplasia. A novel gel-forming system was demonstrated as an effective hemostatic sealing agent. Plans are in place to explore further these three segments to verify these findings following modified protocol and larger number of animals. This is to be followed by development and scale-up studies of at least two systems.

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PRINCIPAL INVESTIGATOR: Shalaby W. Shalaby, Ph.D.

CONTRACTING ORGANIZATION: Poly-Med, Incorporated
Anderson, South Carolina 29625

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L. W. Smalley 7/25/97

PI - Signature Date

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A. INTRODUCTION

Many approaches are being used for treating traumatic and burn wounds such as those encountered in battlefield injuries and burns. However, constraints such as infections, excessive bleeding and/or extreme tissue sensitivity made the treatment of these wounds especially challenging. Thus, the primary objective of this nine-month program is to develop a bioabsorbable (or simply absorbable) hemostatic tissue adhesive with most, if not all, of the following attributes: (1) it can be extruded easily from a syringe as a viscous liquid formulation; (2) the extruded liquid adheres to the tissues and provides sufficient bond strength to keep approximated ends at the wound site in position during healing; (3) the extruded liquid transforms into a gel form at an irregular wound site to allow for 2-4 week residence time and modulates the oxygen and water vapor transmissions; (4) the extruded system before and after gel formation should be mechanically and chemically compatible with injured tissue and any exposed nerve endings; (5) the formulation can be used for the controlled delivery of antibiotics such as vancomycin; and (6) the selected formulations do not interfere with, and preferably accelerate, wound healing. As a secondary objective, the developed formulations can eventually be used clinically to deliver growth factors for accelerated wound healing The objectives of this program were (1) the preparation of several candidate liquid polymeric systems from selected, newly synthesized proprietary gel-forming absorbable polymers; (2) screening the candidate systems for gel-formation, hemostasis and adhesion to animal tissue; (3) evaluation of a selected candidate from "2" for *in vitro* absorption and release profile of vancomycin; and (4) evaluation of selected candidates for efficacy as a hemostatic tissue adhesive using the proper animal models.

However, these objectives were extended to include (1) studying the effect of a selected gel-former, with and without, vancomycin, or the tetrapeptide, RGDS; and (2) investigating the effect of inorganic adjuvants on the gel-former as a hemostatic agent. More specifically, the program was directed to study the efficacy of selected gel-formers with and without bioactive agents or adjuvants on (1) healing and strength regain of an incisional skin wound; (2) healing of a skin burn wounds; and (3) as a hemostatic scaling agent (HSA).

B. SUMMARY OF RESULTS AND SIGNIFICANCE

In general, results of the reported studies demonstrate the feasibility of using effectively the gel-former systems (1) to promote incisional wound healing; (2) in the treatment of burn wounds; and (3) as hemostatic agents. More specifically, (1) a number of gel-former formulations were prepared and screened for uses (1) in conjunction with metallic skin staples to approximate an incisional skin wounds in hairless rats with about half the normal number of staples--results show that the gel-formulation itself can effect a noticeable increase in wound strength regain and a reduction in scar formation at 3-weeks post-operatively, as compared with a full staple line and gel-formulations with a vancomycin or RGDS; (2) in promoting the repair/healing of burn wounds using a newly developed hairless rat model--results indicate that at 14 or 21 days a gel-former alone is effective in improving the healing process. Specific attributes can also be associated with formulations containing vancomycin or RGDS; and (3) gel formulations containing ferric chloride are most effective as hemostatic sealing agents for lacerated rabbit liver, as compared to a placebo or a calcium acetate formulations.

Collectively the results of the completed studies signal two ready-to-develop systems for use in wound repair and hemostasis, namely, the placebo gel and the ferric chloride-containing gels, respectively. Additional studies will be required to maximize the gel-former system for use in treating burn wounds and particularly infected ones. This will entail not only formulation development, but a refinement of the animal model for a higher degree of differentiation of the effect of experimental candidate systems.

C. MATERIALS AND METHODS

C.1. Materials--Monomers, pre-polymers, and key chemical reagents used in this segment of the program were purchased from suppliers listed below.

Chemical	Supplier
Arg-Gly-Asp-Ser (RGDS)	Sigma Chemical
Calcium Acetate	Sigma Chemical
Glutaric anhydride	Aldrich Chemical Company
Glycolide	NORAMCO
dl-lactide	Purac
Polyethylene Glycol 400	Aldrich Chemical Company
Polyethylene Glycol 1000	Aldrich Chemical Company
Stannous octoate	Sigma Chemical
trimethylene carbonate	Boehringer Ingelheim
vancomycin hydrochloride	Sigma Chemical

C.2. Methods

C.2.1. Polymer Synthesis and Characterization--General Polymerization Method--All copolymers used in this segment of the program were prepared by end-grafting polyethylene glycol 400 (PEG-400) or polyethylene glycol 1000 (PEG-1000) with a mixture of glycolide and either dl-lactide or trimethylene carbonate in the presence of a catalytic amount of stannous octaote. The PEG-400 or PEG-1000 was used as the initiator for the ring-opening polymerization. The ring-opening polymerization (end grafting) was conducted and the final product was isolated as described by Shalaby (1997).

Polymer Characterization--Polymers made as described in Section C.2.1. were characterized for (1) chemical identity using FT-infrared (FTIR) spectroscopy--liquid polymers (neat cast from acetone) were analyzed on a Perkin-Elmer Paragon 1000; (2) composition of the copolymeric chains using nuclear magnetic resonance (NMR, both proton and ^{13}C)--the polymers were examined in $CDCl_3$ on a Bruker -300 NMR spectrophotometer; (3) molecular weight and molecular weight distribution of the polymers by gel permeation chromatography (GPC)--the polymers were analyzed as solutions in tetrahydrofuran on a Waters Associate GPC unit; and (4) thermal transitions at or above room temperature using differential scanning calorimetry (DSC)--about 5 mg samples were cooled to -20° and then heated under nitrogen to 150°C at 10°/min heating rate on a Perkin-Elmer DSC-6. A syringe with an 27 gauge needle was used to determine, qualitatively, the fluidity of the individual polymers or their mixtures at 25°C and 37°C.

Preparation of and Characterization of Carboxy-Terminated Gel-Formers--A liquid gel-former made by end-grafting mixtures of dl-lactide/glycolide mixtures onto liquid PEG-400 was used. The liquid polymer, which is hydroxy-terminated, is treated with an equivalent amount of glutaric anhydride to esterify the end-groups and form carboxylic groups at both ends of the chain. The reaction is carried out by heating the mixture at about 100°C for 30 min., 110°C for 40 min, then at 120°C for 40 min. The product was then heated under reduced pressure (about 0.1 mm Hg) at 120° to remove traces of unreacted anhydride. The presence of carboxylic end-groups was confirmed by titration in conjunction with IR spectroscopy. Molecular weight and molecular weight distribution were determined by GPC.

Drug Loading into Gel Formers--Mixed gel formulations were prepared from primary gel formers under aseptic conditions in a laminar flow hood. The required polymers were measured into a 60 cc syringe, mixed with a variable speed motor for five minutes, and then loaded into 1 cc or 5 cc syringes for animal testing. Likewise, gel formulations containing vancomycin, RGDS, ferric chloride, or calcium acetate were prepared by mixing under aseptic conditions. These too were loaded into 1 cc or 5 cc syringes for animal testing.

C.2.2. *In Vitro* Screening of Candidate Gel-Formers--Different combinations of the original gel-formers having different chain structures, hydrophilicity, and solubility were evaluated and rated on a scale of 1 - 5 in the different test categories using 5 and 1 as the most and least desirable, respectively.

Gelation Time--A 0.5 ml aliquot of the gel-former was extruded from a syringe needle into 5 ml buffer solution at pH 7.2 and 25°C and time required to form a coherent 3-dimensional mass was noted.

Adhesive Property--At this stage of the program, the adhesive property was determined in terms of the ability of the gel-former to adhere to the walls of a glass vial at 25°C in presence of phosphate buffer at pH of 7.2. Thus, a 0.5 ml of gel-former was extruded from a syringe needle into 20 ml of a buffer solution, left to equilibrate for 5 min. The vial is then shaken for 10 seconds and resistance to dislocation of the gel mass from the bottom of the vial is used as a measure of its adhesive property.

C.2.3. *In Vitro* Absorption and Release Studies of Drug Loaded Formulations--*In Vitro* Absorption--Relative rates of absorption of four selected gel-formulations containing 5 and 10 percent vancomycin were determined in terms of time required for practically complete dissolution at 50°C in a phosphate buffer solution (0.5 g. formulation in 50 ml buffer) in a shaker incubator at 50°C.

Release Profile of Drug-Loaded Formulation--In a typical experiment, the gel-former is mixed with 5 or 10 percent vancomycin. The drug-loaded polymer is transferred to a continuous-flow cell attached to a peristaltic pump. The buffered phosphate solution was passed tangentially by the surface of the drug release systems. Samples of the effluent buffer were collected at regular intervals over a period of 15 days. The drug content of vancomycin in these samples was determined using an HPLC method developed at Poly-Med. This method calls for use of an acetonitrile/water mobile phase and a C_{18} column.

C.2.4. Animal Studies--Adhesive Skin Wound Augmentation (ASWA)--CD hairless rats were injected with 0.05 mg/kg Buprenex and 0.50 mg/kg Acepromazine maleate to initiate anesthesia. Once initiated, anesthesia was maintained via 2% isofluorane inhalation. Each rat was shaved and

then scrubbed alternately with Nolvasar scrub and isopropyl alcohol. Two 5 cm long incisions were made on the back of each rat 2 cm lateral to the dorsal midline, beginning at the level of the T-11 vertebra. One incision was closed using nine metal staples placed 0.5 cm apart, the traditional standard for wound healing. The second incision was closed using four metal staples placed 1 cm apart with 1 cc gel applied down the length of the incision. Two formulations were tested in the first segment of the study: a placebo gel former and a gel former containing 2% vancomycin. Each test group consisted of six rats. A follow-up study was conducted on two rats using a gel former with 1 mg/ml RGDS added.

At one week post-op, several rats had removed some of their staples by chewing. Within the first week, two rats removed enough staples to leave gaping wounds which could not heal properly. These rats were therefore euthanized. All staples were removed from the remaining rats by a veterinarian at ten days post-op. These rats were euthanized in a pre-charged CO_2 chamber at three weeks. Immediately post-mortem, the skin about the incision was dissected, immersed in saline, and taken to the laboratory for mechanical testing.

Guided by a previously reported procedure (Linden & Shalaby, 1996), each skin sample was cut into five test strips as shown in Figure 1, and the dimensions of each sample was measured. The healed incision strength was measured using a Satec T10000 mechanical testing apparatus. The skin was secured into the grips of the machine, and the force to pull the wound apart was measured at a displacement rate of 50 mm/min.

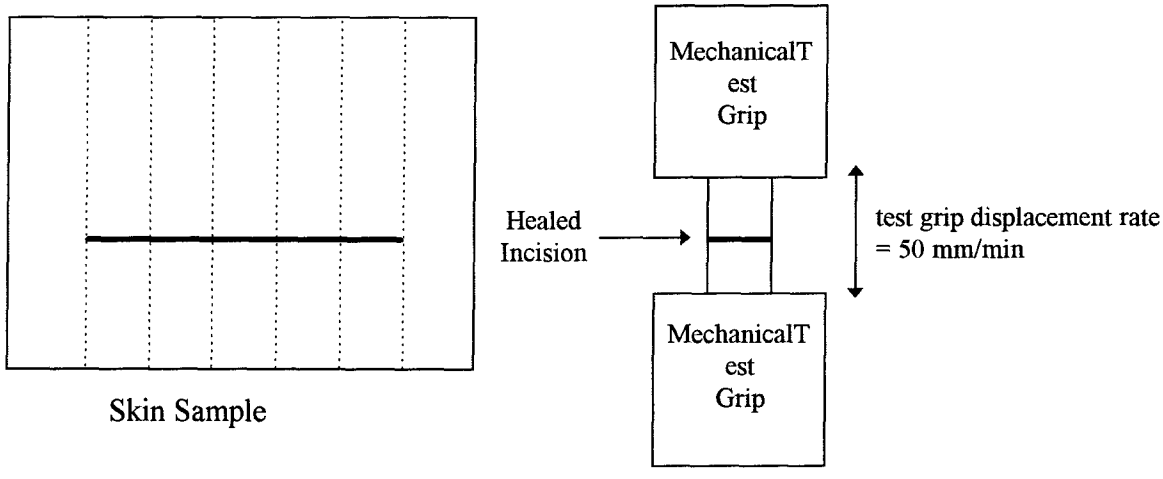

Figure 1. Sectioning and Testing of Skin Wound

Burn Wound Healing (BWH)-- A contact burner was developed for this study which consisted of a 1 cm^2 smooth, copper plate attached to the end of a soldering iron. A rheostat was used to control the burner temperature. A pilot study was conducted on one CD hairless rat to develop a burning protocol. Ten burns were created on the back of this rat using different burner temperatures and application times. Histological evaluation of these burns indicated that second degree burns could be effected by heating the burner to 100°C, quenching in boiling water for ten

seconds, and applying to the rat for ten seconds. This protocol was followed for all subsequent studies.

CD hairless rats were injected with 0.05 mg/kg Buprenex and 0.50 mg/kg Acepromazine maleate to initiate anesthesia. Once initiated, anesthesia was maintained via 2% isofluorane inhalation. Each rat received two burns according to the established protocol described above. Burns were inflicted 1 cm lateral to the dorsal midline just below the shoulder blades. Three to five minutes after burning, 1 cc of a gel former was applied to one burn on each rat. Four different gel formulations were evaluated, a placebo gel former, a gel former containing 0.2% vancomycin, a gel former containing 1 mg/ml RGDS, and a gel former containing 3 mg/ml RGDS. All formulations were tested on three rats with the exception of the gel former containing 3 mg/ml RGDS which was tested on one rat. An Elizabethan collar was placed around the neck of each rat to prevent it from disturbing the wound site.

Wounds were measured and photographs were taken at the time of burn infliction and weekly thereafter. Selected photographs were submitted to the Clemson University Bioengineering Department for computerized image analysis to compare the degree of healing in treated and untreated burn wounds. Rats were euthanized in a CO_2 precharged chamber at three weeks post-op. Each burn wound was dissected out, stapled to cardboard to maintain the wound shape, and preserved in 10% formalin. Samples were submitted for histological slide preparation using hematoxylin and eosin and Masson's trichome stains. Histological evaluations were conducted by Pathology Associates International.

Hemostatic Sealant System (HSS)--New Zealand white rabbits were anesthetized via 2% isofluorane inhalation. The rabbit liver was exposed, an incision 2-3 cm long was created, and 3-4 cc of a selected gel former was applied. The capacity to stop bleeding was observed, and photographs were taken to record the performance of each gel formulation. On average, two incisions were tested per rabbit. A total of twenty-one different gel formers were tested on 13 rabbits. The tested hemostatic agents consisted of gel formers of varying viscosity with ferric chloride or calcium acetate added to selected formulations. At the conclusion of the experiments, the rabbits were euthanized via 4% isofluorane inhalation.

D. EXPERIMENTAL RESULTS AND DISCUSSION

D.1. Synthesis and Characterization of Primary Gel-Formers--Six primary gel formers having the composition shown in Table I were prepared. Their composition and molecular dimensions coincided with those expected. With the exception of GF-F, all primary gel formers consisted of lactide and glycolide with either PEG-400 or PEG-1000. GF-F consisted of PEG-400 with trimethylene carbonate and glycolide.

Table I. Compositions of the Primary Gel-Formers and Mixtures Thereof

Primary Gel-Formers	GF-A	GF-B	GF-C	GF-D	GF-E*	GF-F**
•Composition of Primary GFs						
PEG: Type (wt%)	400(85)	1000(30)	400(30)	400(20)	400(20)	400(20)
Polyester: L/G (%)	60/40(15)	80/20(70)	60/40(70)	60/40(80)	60/40(80)	60/40(80)

Table 1 Cont'd.

Primary Gel-Formers	GF-A	GF-B	GF-C	GF-D	GF-E*	GF-F**
•Composition of Mixtures made of primary GFs (%GF)						
GF-I	30	70	—	—	—	—
GF-II	38	—	62	—	—	—
GF-III	30	—	—	70	—	—
GF-IV	40	—	—	60	—	—
GF-V	17	—	27	56	—	—
GF-VI	10	—	10	—	80	—
GF-VII	—	—	25	75	—	—
GF-VIII	30	—	—	—	—	70

* Carboxy-Terminated ** PEG 400/(TMC/G)

Viscosity and solubility characteristics of the individual primary GFs were considered less than optimal for use, separately, in any of the segments of Phase I. This is because each of these GFs displayed relatively extreme property in terms of hydrophilicity, gelation time, adhesiveness and/or absorbability. Therefore, it was decided to prepare and study the properties of selected mixtures of the primary GFs as planned in the original program proposal. GF-D was carboxy-terminated (to form GF-E), and its equivalent weight, as determined by titration, was comparable to the expected value of about 1200 Da.

D.2. Preparation and Properties of Mixed Gel-Formers--Eight combinations of the primary gel-formers were prepared as described in Table I. Based on the properties of these mixtures, in terms of gelation time and adhesion to polar substrates (e.g., Pyrex glass surface), five most promising systems (III, V, VI, VII, and VIII) were chosen for conducting additional studies (as in Section C.3.)

D.3. Preparation Properties and Selection of Candidate Formulations for *In Vivo* Studies--Effect of Composition on Gel Quality and Drug Release Profile--Gel-formers (GF-III, GF-V, GF-VI, GF-VII, and GF-VIII) described in the previous section were selected to load with 10% vancomycin; an additional sample of GF-VII was loaded with 5% vancomycin. The release profile of these systems over 15 days was monitored and the results indicate that (1) using GF's based on PEG-400 copolymers are most promising in terms of more controlled, slow release; (2) having high fractions of the high molecular weight GF-D is essential for attaining acceptable gel mechanical integrity; (3) incorporating GF-A and/or GF-B enhances the adhesive property of the gel; (4) using carboxy-terminated components do not slow the drug release rate; and (5) having a high drug concentration of 10% does not compromise the flow properties or the drug release profile of the formulation.

Effect of Composition on Relative Absorption--Results of the absorption study in a phosphate buffer at pH 7.2 and 50°C indicate that the absorption of the five formulations having 10% vancomycin decrease in the following order:

GF-III > GF-V > GF-VII > GF-VIII > GF-VI

Identification of Different Formulations for the In Vivo Study and Rationale--For the **adhesive skin wound augmentation** study, the following gel formers were tested for reasons described below: (1) The polymeric components of GF-V were selected for the placebo formulation due to their adhesiveness and gel forming qualities. (2) Vancomycin loading of a relatively high concentration (compared to common topical formulations) of 2% was selected to maximize the effect of this antibiotic on the healing process. It was expected that antibiotics may interfere with the wound healing process. However, this protocol was followed since a key potential clinical application of the gel formers is expected to be associated with infected wounds (as in battlefield situations). The role of high concentrations of antibiotics needed to be explored in a quantitatively evaluated animal model. (3) Arg-Gly-Asp-Ser (RGDS), an oligopeptide carrying an adhesion site of the adhesion protein fibronectin (i.e., tripeptidyl sequence RGD), was selected in order to study its effect on modulating the wound healing process. The role of RGDS in wound healing and relevant biological events has been noted by a number of authors (Garcia et al., 1996; Holland et al., 1996; Streeter & Reese, 1987). The RGDS was tested at a concentration of 2 mg/ml in the gel former.

To determine the effect of the composition of both the polymeric carrier and active components on **burn wound healing**, the following gel formers were chosen for the cited reasoning: (1) The polymeric components of GF-V were selected for their optimum adhesiveness and gel-forming quality. In addition, using the same system in wound augmentation and burn wound healing will allow for a comparative evaluation of the role of the placebo formulation in two different procedures. (2) A 0.2% loading of vancomycin was used to minimize any possible compromise of the healing process. This concentration is comparable to those used in many topical antibiotic applications. Such loading represents only 10 percent of the concentration used in the adhesive and wound augmentation procedure, since the effect of the gel-formulation in the burn study will be qualitatively assessed and stressing the biological system was not necessary. (3) A 1 mg/ml loading of RGDS was chosen as a moderate- to high-dose. Meanwhile, in an attempt to assess the effect of RGDS concentration , a 2 mg/ml dose was also used. Also, a 2mg/ml dose was used to allow for a comparative evaluation of the role of the formulation in both the tissue adhesive and burn procedures.

Toward determining the efficacy of gel-formers as **hemostatic sealing agents**, twelve gel formers were initially tested. Of these, two basic polymeric carriers were chosen for the following reasons: (1) Placebo formulations of GF-II and GF-VIII were tested to determine the ability of the gel formers to spread out quickly and cover the incision site. (2) Both placebo carriers were mixed with calcium acetate and ferric chloride to determine the effect of divalent and trivalent ions on hemostatic properties.

D.4. Adhesive Skin Wound Augmentation Study--This study was pursued as per the animal protocol flow chart in Appendix B. Main segments of the study and the pertinent results are summarized below.

D.4.1. Preparation of Placebo and Active Gel-Formers--GF-V was selected for both placebo and active formulations in this study. In the first segment of the study, GF-V and GF-V with 2% vancomycin were tested on six rats each. In a follow-up study, GF-V with 2mg/ml RGDS was tested on two rats.

D.4.2. Animal Surgery and Results--Subjects--Twelve CD hairless rats, six per group, were used in the initial study. In a follow up study, two CD hairless rats were used. Each rat was injected with 0.05 mg/kg Buprenex and 0.50 mg/kg Acepromazine maleate. Once the rats appeared sedated, anesthesia was maintained with 2% isofluorane.

Procedure-- Each rat was shaved and then scrubbed alternately with Nolvasar scrub and isopropyl alcohol. Two 5 cm long incisions were made on the back of each rat 2 cm lateral to the dorsal midline, beginning at the level of the T-11 vertebra. One incision was closed using nine metal staples placed 0.5 cm apart, the traditional standard for wound healing. The second incision was closed using four metal staples placed 1 cm apart with 1 cc gel applied down the length of the incision. The rats were placed in cages lined with paper for recovery from anesthesia. Buprenorphine was administered via subcutaneous injection every 8 to 12 hours for 24 hours after surgery. In the initial study with 12 rats, no collars were used. In the 2 rat follow-up study, Elizabethan collars were placed on the rats before recovery from anesthesia to prevent them from mutilating the wound site.

Observations--From the time of surgery until the staples were removed, the control incision with nine staples looked puckered and bunched up compared to the test side which appeared more flat and even.

In the first week of the initial study on twelve rats, two rats, one from each group (GF-V and GF-V with 2% vancomycin), mutilated their incision sites such that they could not heal. These rats were euthanized, and skin samples were dissected out and used to validate mechanical test methods used at the end of the study. In the follow-up study, Elizabethan collars were put on the rats to prevent them from reaching the incision sites.

Ten days after surgery, all staples were removed. All incisions, both test and control, appeared to be healing properly. Superficial scabs were noted on several rats at this time. There was no apparent correlation between the location of the scabs and either control or test incisions in the rats. In general, the scabs were located on the middle of the rats' backs, between the two incisions. These scabs never worsened but persisted the entire duration of the study.

At twenty-one days after surgery, a mass was found on one of the rats which received the GF-V treatment. The mass was located near the rat's abdomen, far from the incision sites.

Euthanasia and Preparation for Wound Breaking Strength Measurement--All rats were euthanized in a CO_2 pre-charged chamber except for two euthanized by heart puncture as noted below. Skin was collected from around the incision sites. Upon dissection of the skin, all scabs were noted to be very superficial with increased vascularity underneath. Each skin sample was placed in a specimen jar containing saline. The mass found in one rat, as noted in the observations above, was removed and placed in formalin for histopathology.

Two rats from the initial study, one without any scabs at all and one with the largest scab, were anesthetized with a ketamine/xylazine mixture and euthanized via heart puncture. Blood was collected from each and sent away for a complete blood count (CBC). A culture swab was taken of the scab on the one rat, and tissue samples were taken from both rats for histopathology.

Wound Strength Testing--All wound strength testing was conducted within 12 hours of sacrifice. Skin samples were taken to the laboratory for testing where each incision was cut into five test samples measuring about 1 x 6 cm^2. The width and thickness of the healed incision were measured for each specimen.

Samples from the initial study were tested for wound strength using a Satec T10000; samples from the follow-up study were tested on an MTS 858 Minibionix. In both studies, the samples were tested at a ram rate of 50 mm/min until failure, defined as an 80% drop in force. The data were recorded and a graph of load versus displacement was generated. The wound strength was calculated as the maximum force applied over the area of the incision site.

Results of Wound Strength Testing--The results of the wound strength testing are shown in Figure 2 below. Because the initial study and follow-up study samples were tested on different mechanical testing machines, the data from the two cannot be compared. Data in the figure for GF-V and 2% Vancomycin should be compared to Control 1 while data for 2 mg/ml RGDS should be compared to Control 2.

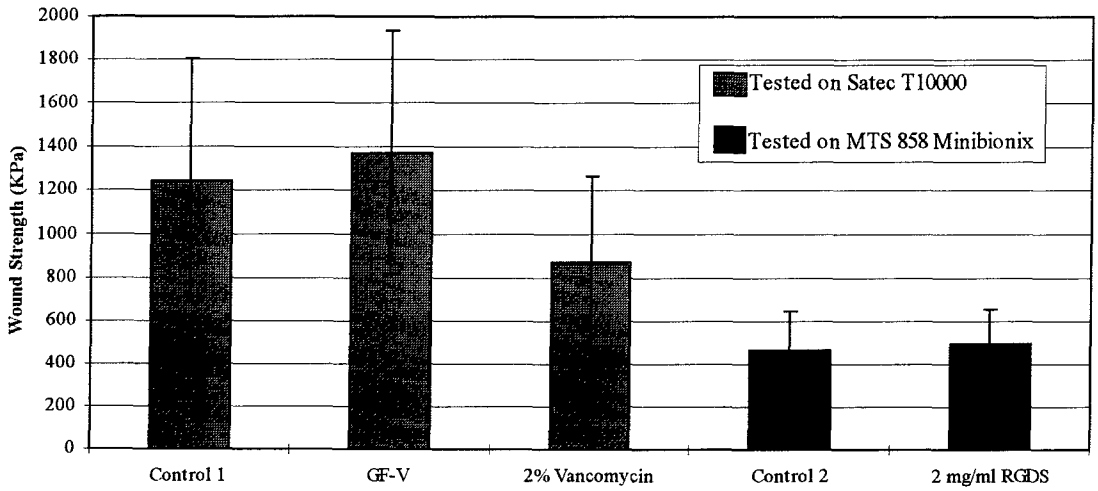

Figure 2. Wound Strength

Results of Histopathology, CBC, and Culture Swab--Histopathology of the mass found in one rat was performed by the Clemson Animal Diagnostic Laboratory. Microscopic exam of the mass revealed a well demarcated mass composed of a uniform population of epithelial cells forming tubules. The cells had hyperchromatic nuclei and scant cytoplasm; mitotic figures were common. The mass was diagnosed as tubular adenoma.

CBC of the blood samples from two rats showed no abnormalities. Skin and scab samples were submitted to the Clemson Animal Diagnostic Laboratory. No abnormalities were found in the skin samples. The scab sample was diagnosed as having necrotizing epidermatitis with chronic diffuse dermatitis and a superficial necrotic crust. The swab taken of one of the scabs was also submitted to the Clemson Animal Diagnostic Laboratory. The swab culture revealed the presence of staphylococcus coagulase (-) and morganella morganii.

D.5. Burn Wound Study--This study was pursued as per the animal protocol flow chart in Appendix C. Main segments of the study and the pertinent results are summarized below.

D.5.1. Preparation of Gel Formers--GF-V was selected for both placebo and active formulations in this study. Using GF-V for both wound augmentation and burn wound healing allowed for a comparative evaluation of the role of the gel former in the two procedures. In addition to the placebo, three different active formulations were tested which contained 0.2% vancomycin, 1 mg/ml RGDS, and 3 mg/ml RGDS. The formulations were mixed under aseptic conditions in a laminar flow hood and packaged in 1 cc syringes for animal surgeries.

D.5.2. Animal Preparation and Treatment--Subjects--One CD hairless rat was used in a pilot study to develop a burn protocol. Once the burn protocol was established, ten CD hairless rats were used to evaluate the performance of gel formers in burn wound healing.

Development of Contact Burn Protocol--A pilot study was conducted on one CD hairless rat to develop a burning protocol. The rat was injected with 0.05 mg/kg Buprenex and 0.50 mg/kg Acepromazine maleate to initiate anesthesia. Once initiated, anesthesia was maintained via 2% isofluorane inhalation. Ten burns were created on the back of this rat using different burner temperatures and application times. The rat was then euthanized via 4% isofluorane inhalation. Burn samples were dissected out and preserved in formalin for histological evaluation.

Results of Pilot Study--Burn samples were submitted to the Clemson University Bioengineering Department for histological evaluation. Results indicated that the contact burn protocol led to second degree to severe third degree burns. According to the report, **second degree burns** could be effected by heating the burner to 100°C, quenching in boiling water for ten seconds, and applying to the rat for ten seconds. This protocol was thus followed for all subsequent studies.

Contact Burn Procedure--CD hairless rats were injected with 0.05 mg/kg Buprenex and 0.50 mg/kg Acepromazine maleate to initiate anesthesia. Once initiated, anesthesia was maintained via 2% isofluorane inhalation. Each rat received two burns according to the established protocol described above. Burns were inflicted 1 cm lateral to the dorsal midline just below the level of the shoulder blades as shown in Figure 3. Three to five minutes after burning, 1 cc of a gel former was applied to one burn on each rat as illustrated in Figure 4. Four different gel formulations were evaluated, GF-V, GF-V with 0.2% vancomycin, GF-V with 1 mg/ml RGDS, and GF-V with 3 mg/ml RGDS. All formulations were tested on three rats with the exception of the gel former containing 3 mg/ml RGDS which was tested on one rat. An Elizabethan collar was placed around the neck of each rat to prevent it from disturbing the wound site.

Observations--Wounds were measured and photographs were taken at the time of burn infliction and weekly thereafter. At one week, all rats had dry skin due to the fact that the Elizabethan collars prevented them from grooming. Because it was important that the burn wound sites not be disturbed, the collars were left in place. At this time, scabs were beginning to develop at the wound sites as shown in Figure 5. At two weeks, all burns had well developed scabs which were beginning to rise and peel off in some cases as shown in Figure 6. By three weeks, most wounds had a healed pinkish zone about the wound as shown in Figures 7 and 8.

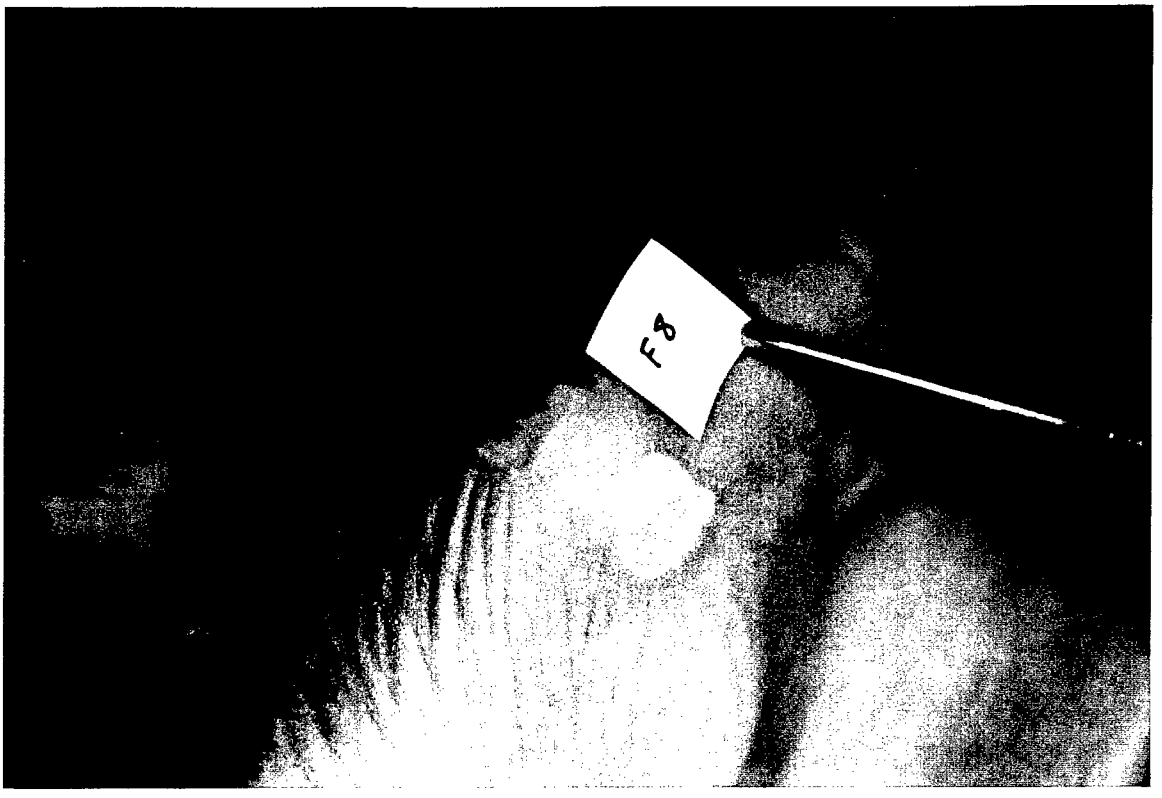

Figure 3. CD Hairless Rat after Infliction of Two 1 cm^2 Burns

Figure 4. Application of Gel Former to One Burn

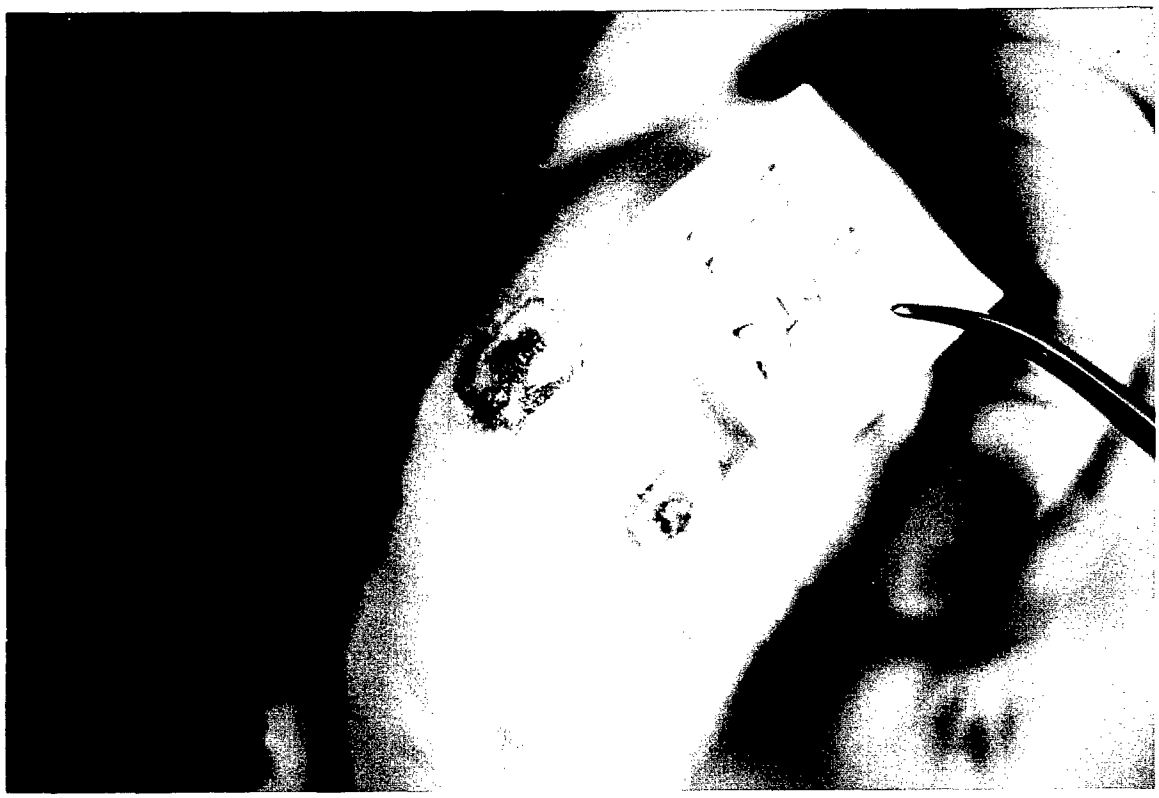

Figure 5. Wounds 8 days Post-Burn (Treated burn on the right.)

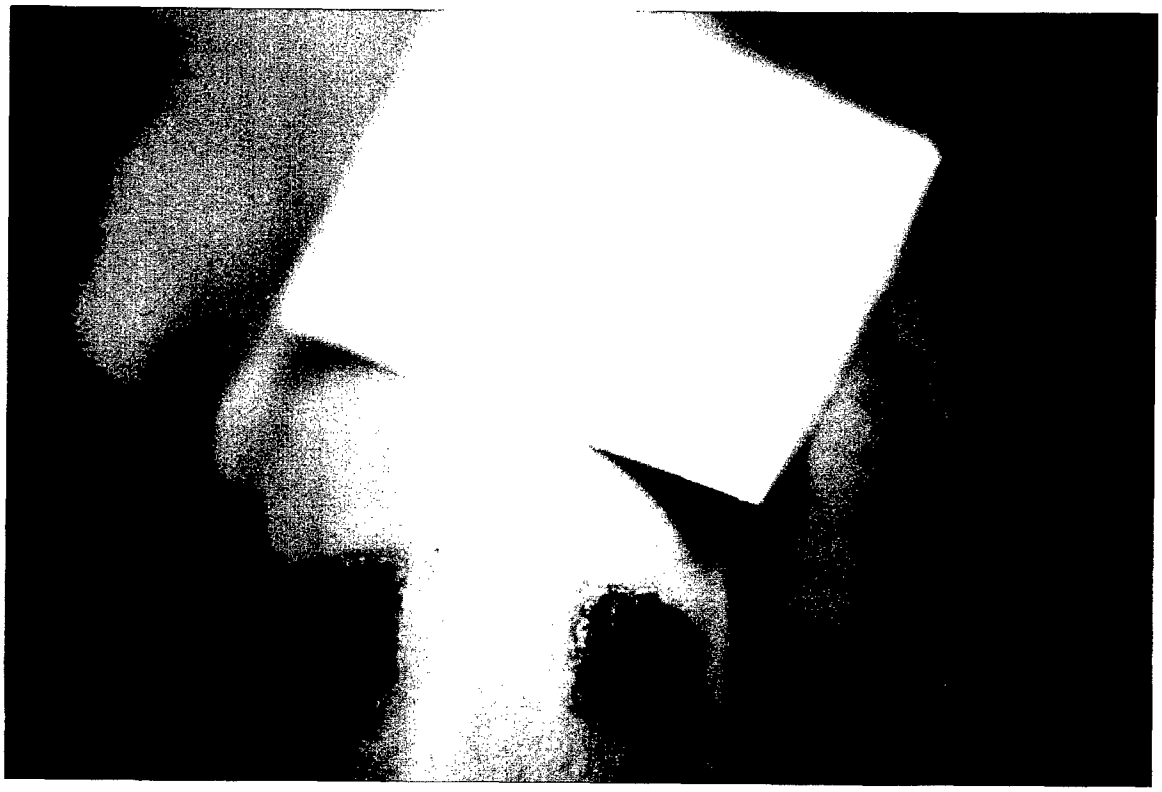

Figure 6. Wounds 14 days Post-Burn (Treated burn on the right.)

Figure 7. Treated Wound 21 days Post-Burn

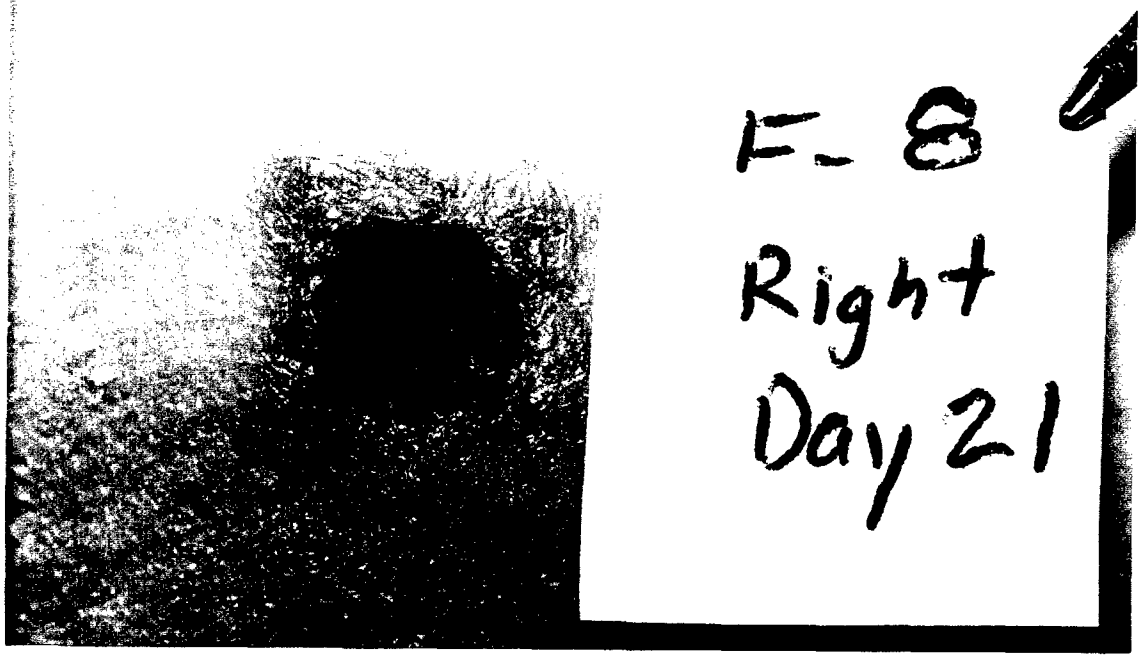

Figure 8. Control Wound 21 days Post-Burn

Euthanasia and Preparation for Wounds for Histology--Rats were euthanized in a CO_2 precharged chamber at three weeks post-op. Each burn wound was dissected, stapled to cardboard to maintain the wound shape, and preserved in 10% formalin. Samples were submitted for histological slide preparation using hematoxylin and eosin and Masson's trichome stains. Histological evaluations were conducted by both Pathology Associates International and the Clemson University Bioengineering Department.

D.5.3. Histological Evaluation and Imaging Analysis of Healing Burn Wounds--Imaging analysis was conducted on photographs of typical healing wounds which have been treated with the placebo (with gel-former only) and untreated controls photographed at the 21 day period. Using an arbitrary calibration, two areas were measured on each photograph--the original area of the wound and the area of the remaining scab. Subtracting these two values was used to calculate the area of the wound that has healed. However, no significant difference could be determined between the placebo-treated and untreated control specimens. Accordingly, more emphasis was placed on the histological evaluation of several healing wound specimens that were harvested at 21 days, post-treatment. Tentative results of this evaluation are summarized in Table II. Although no definitive conclusions can be drawn as to the overall effect of any particular burn treatment as compared to the untreated controls, each of the treated burns displayed at least one specific positive feature. Among the different burn wounds and particularly in comparison with untreated controls, (1) the placebo gel-former resulted in a relatively thin serocellular crust, a maximum area of epithelialization, and a minimum width dermal fibroplasia; (2) the vancomycin formulation was associated with a minimum ulcer width; and (3) a wound treated with RGDS formulation did show an increase in epithelialization. These tentative results are in concert with the observed positive effects of the placebo formulation on wound strength regain discussed above.

Table II. Histological Evaluation and Imaging Analysis of Healing Burn Wounds*

Treatment	Ulcer Width (mm)	Serocellular Crust (mm)	Dermal Fibroplasia Width (mm)	Dermal Fibrosis Width (mm)	Dermal Fibrosis Thickness (mm)	Epithelialized Area %
Untreat. Control	5.9	0.80	8.0	8.5	0.70	53
Placebo	3.0	0.26	2.5	8.5	0.80	80
0.2%vancomycin	2.0	0.40	5.0	6.0	0.60	42
1 mg/ml RGDS	3.1	0.70	7.0	7.5	0.70	74

* Using 21 day specimens.

D.6. Hemostatic Sealing Agents--This study was pursued as per the animal protocol flow chart in Appendix D. Main segments of the study and the pertinent results are summarized below.

D.6.1. Preparation of Gel Formulations--GF-II and GF-VIII were selected for use in this study with and without 5% and 10% of calcium acetate or ferric chloride. These multivalent salts were mixed first with the low viscosity component of GF-II or GF-VIII (GF-A) prior to preparing the respective final formulations. The GF-VIII was distinguished for its higher molecular weight, fluidity, and tendency to adhere to soft tissues as compared to GF-II.

D.6.2. Effect of Composition on Hemostasis and Tissue Sealing--Application of placebo gel-formers appears to result in hemostasis through the formation of a barrier membrane. The latter

seems to lack the mechanical integrity of a good sealant (Sierra & Saltzer, 1996). Adding multivalent ion coagulating adjuvants, such as ferric chloride, led to a timely hemostasis through formation of a barrier membrane with excellent mechanical integrity. The overall performance of the 5% exceeded that of the 10% formulations. Figures 9 and 10 depict a lacerated liver before and after treatment with a gel-former containing soluble ferric chloride. A similar effect could not be achieved upon replacing ferric chloride with calcium acetate. Replacing GF-V with the more tissue adhering GF-VIII in formulations containing about 5% to 10% $FeCl_3$ produced the most effective hemostatic sealants. More specifically, the 5% formulation produced a fast-forming barrier hemostatic sealant which exhibited exceptional mechanical strength while being flexible.

D.7. Problem Areas and Corrective Measures

1. Toward achieving small differences in wound healing of augmented skin wounds, the use of sutures was thought to compromise detection of such differences.

Corrective Measure--East-to-apply metallic staples were used to approximate the wound edges.

2. Two of the augmentation study rats "Picked" most of the staples at 1 and 6 days, post-operatively, leading to wound gaping. These animals were euthanized.

Corrective Measure--Elizabethan collars were purchased and installed on the rats and used in repeating part of the wound repair study and implementing the burn wound healing study.

Figure 9. Untreated Incision

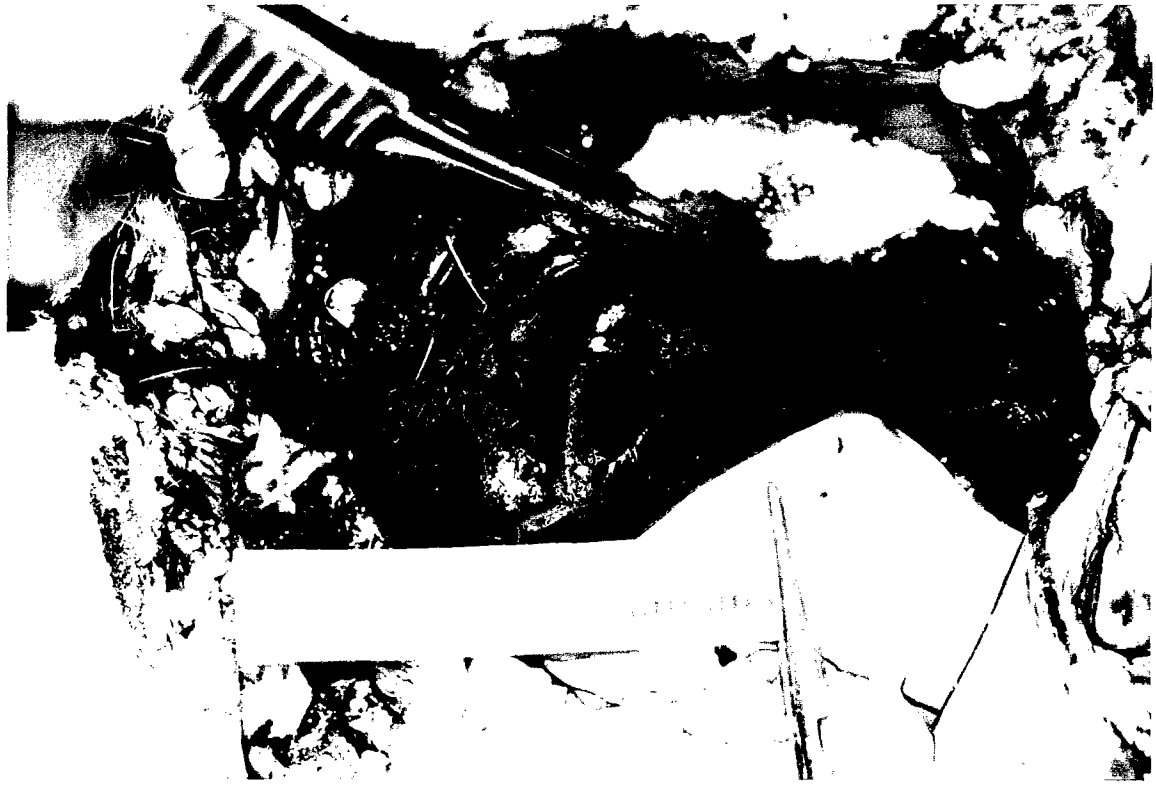

Figure 10. Incision 2 min after Appliction of Gel Former

E. CONCLUSIONS AND RECOMMENDATIONS

E.1. Conclusions--Results of the studies subject of this report can be used to draw the following conclusions.

1. Typical examples of the PEG-based copolyester family of absorbable gel-forming injectable liquid gels-formers can be formulated into useful agents for application in wound management without eliciting adverse tissue reactions.

2. Typical gel-formulations may be used as non-invasive adjuvants to staples and possibly sutures.

3. By using gel-forming adjuvants, the number of staples usually required for wound repair can be reduced significantly, while achieving a discernible increase in wound strength regain and minimum scar formation.

4. The gel-formers can be used as adhering burn covers for positive modulation of the healing process to accelerate the healing process and minimize scar formation.

5. Incorporating ferric chloride as a solution in typical gel-formers provides an adhering hemostatic sealant.

6. The use of high concentrations of antibiotic in the gel-formers may compromise their performance in wound repair.

7. The effect of RGDS on the performance of the gel-formers in wound repair, wound burn treatment or hemostasis was limited.

E.2. Recommendations--Future activities on this program are recommended to entail the following R&D segments.

1. Development of the gel-formers as adhesion **adjuvants for metallic skin staples**.

2. Exploring the use of gel-formers as adhesive **adjuvants for absorbable sutures** particularly those used in plastic surgery.

3. Extending the burn wound study to a third degree sterile and contaminated site, as well as development of an absorbable burn cover formulation for **battlefield and emergency room** patients.

4. Developing a simple ferric chloride gel-former formulation as **hemostatic-sealants** for use for **battlefield and emergency room** patients.

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Appendix A

AN OVERALL ABSTRACT FOR THE THREE ANIMAL PROTOCOLS

Many approaches are being used for treating traumatic and burn wounds such as those encountered in battlefield injuries and burns. However, constraints such as infections, excessive bleeding and/or extreme tissue sensitivity make the treatment of these wounds especially challenging. Thus, the primary objective of this nine-month program is to develop a bioabsorbable (or simply absorbable) hemostatic tissue adhesive with most, if not all, of the following attributes: (1) it can be extruded easily from a syringe as a viscous liquid formulation; (2) the extruded liquid adheres to the tissues and provides sufficient bond strength to keep approximated ends at the wound site in position during healing; (3) the extruded liquid transforms into a gel form at an irregular wound site to allow for 2 to 4 weeks residence time and modulates the oxygen and water vapor transmissions; (4) the extruded system before and after gel formation should be mechanically and chemically compatible with injured tissue and any exposed nerve endings; (5) the formulation can be used for the controlled delivery of antibiotics such as vancomysin; and (6) the selected formulations do not interfere with, and preferably accelerate, wound healing. As a secondary objective, the developed formulations can eventually be used clinically to deliver growth factors for accelerated wound healing. Most pertinent to the three individual protocols is a description of the intended animal studies which can be documented as follows.

Protocol I—Wound Augmentation

The adhesive properties of two gel formulations, one with and one without vancomycin, will be evaluated using a set of 6 rats for each formulation. Two 5 cm skin incisions will be made along both sides of the spine. One incision will be closed using a gel formulation and four staples, and the other will be closed using nine staples. After three weeks, the rats will be sacrificed, and the area of skin about the healed incision will be removed and prepared for testing of wound strength. Staples will be removed prior to testing.

Protocol II—Burn Wounds

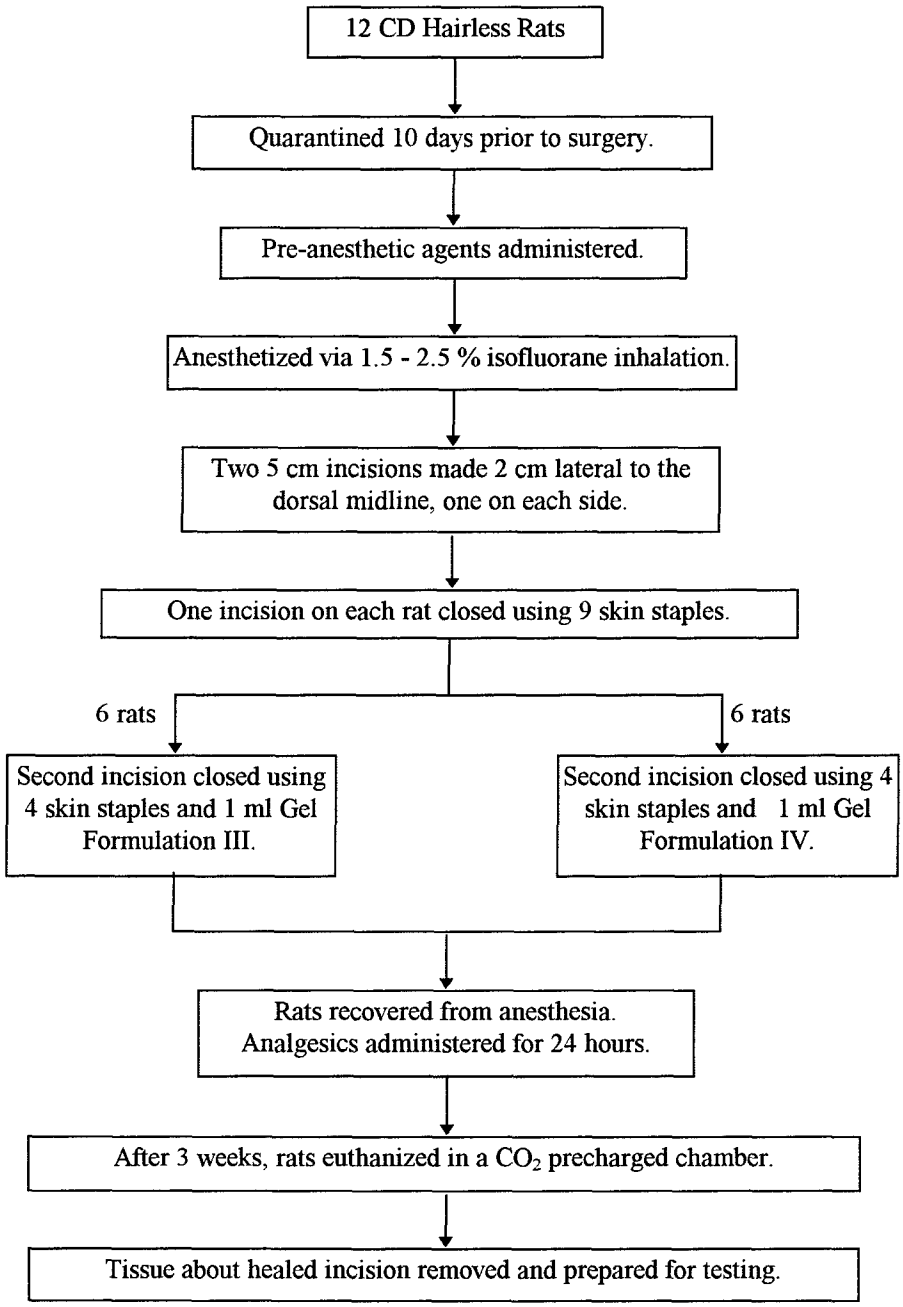
For burn wound evaluation, a full thickness thermal injury will be achieved using a specially designed electrically heated flat plate. Burn wounds will be created at two sides of the rat spine. One burn will be left untreated for control and one will be treated with one of three gel formulations. Nine animals will be used to test the experimental gel formulations, i.e., three animals per gel formulation. The extent of healing over a period of three weeks will be assessed grossly, with reduction in wound area being the main criterion.

Protocol III—Hemostatic Sealing

The hemostatic properties of two gel formulations, one with and one without vancomycin, will be evaluated using a rabbit model where liver lacerations will be created using a scalpel. The ability of the gel formulations to stop bleeding will be assessed grossly in terms of time to stop bleeding.

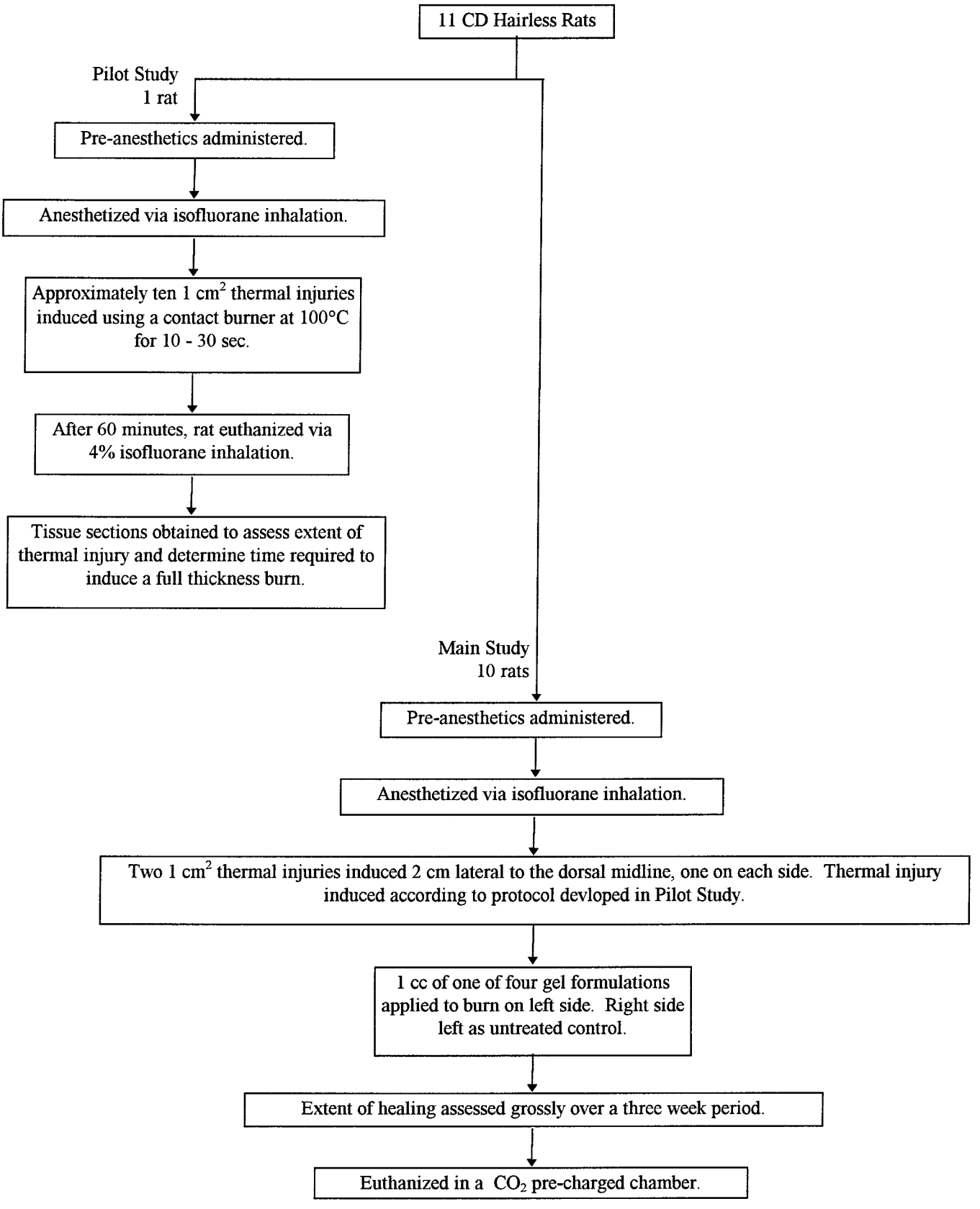
Appendix B

Adhesive Skin Wound Augmentation: Animal Study Flow chart



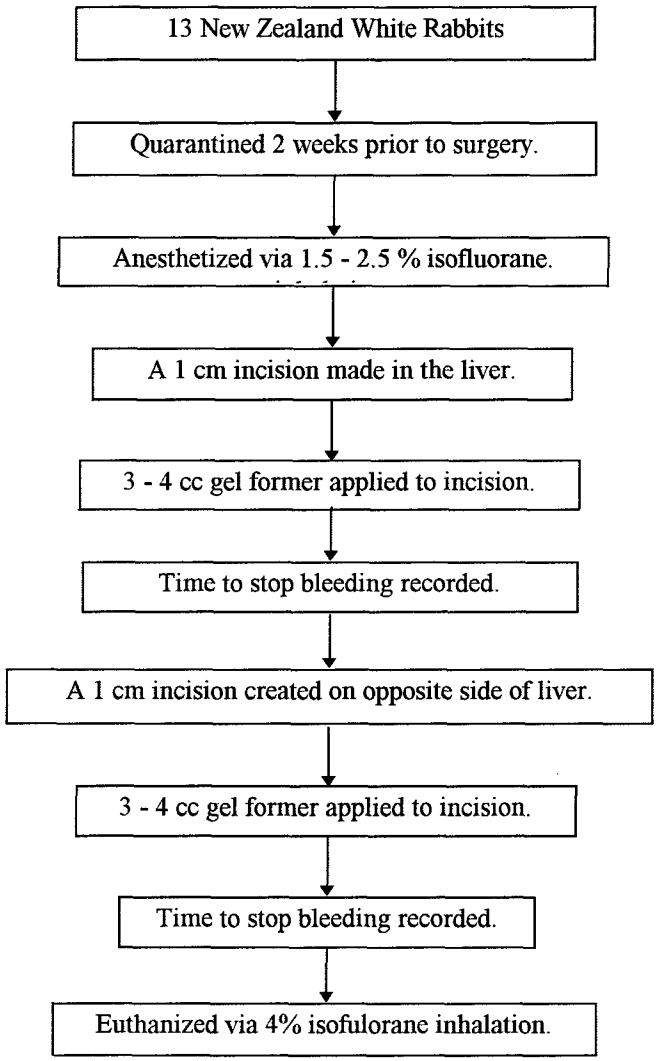
Appendix C

Burn Wound Healing: Animal Study Flow Chart



Appendix D

Hemostatic Sealing Agents: Animal Study Flow Chart



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 10 Aug 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
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DAMD17-92-C-2053	ADB196427
DAMD17-94-C-4022	ADB190750
DAMD17-94-C-4023	ADB188373
DAMD17-94-C-4027	ADB196161 ✓
DAMD17-94-C-4029	ADB190899
DAMD17-94-C-4039	ADB188023
DAMD17-94-C-4024	ADB189184
DAMD17-94-C-4026	ADB187918
~~DAMD17-94-J-4250~~	~~ADB221970~~
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DAMD17-96-1-6241	ADB233224
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DAMD17-91-C-1135	ADB233658 ✓
DAMD17-94-J-4038	ADB232313 ✓
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DAMD17-94-J-4159	ADB232305 ✓
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MCMR-RMI-S
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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or email: judy_pawlus@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management